



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Lgr5(+) gastric stem cells divide symmetrically to effect epithelial homeostasis in the pylorus

Citation for published version:

Leushacke, M, Ng, A, Galle, J, Loeffler, M & Barker, N 2013, 'Lgr5(+) gastric stem cells divide symmetrically to effect epithelial homeostasis in the pylorus', *Cell Reports*, vol. 5, no. 2, pp. 349-56.
<https://doi.org/10.1016/j.celrep.2013.09.025>

Digital Object Identifier (DOI):

[10.1016/j.celrep.2013.09.025](https://doi.org/10.1016/j.celrep.2013.09.025)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Cell Reports

Publisher Rights Statement:

This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Lgr5⁺ Gastric Stem Cells Divide Symmetrically to Effect Epithelial Homeostasis in the Pylorus

Marc Leushacke,¹ Annie Ng,¹ Joerg Galle,² Markus Loeffler,³ and Nick Barker^{1,4,5,*}

¹A*STAR Institute of Medical Biology, 138648 Singapore, Singapore

²Interdisciplinary Centre for Bioinformatics, University Leipzig, 04107 Leipzig, Germany

³Institute for Medical Informatics, Statistics and Epidemiology, 04107 Leipzig, Germany

⁴Centre for Regenerative Medicine, The University of Edinburgh, 47 Little France Crescent, Edinburgh EH16 4TJ, UK

⁵Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, 117596 Singapore, Singapore

*Correspondence: nicholas.barker@imb.a-star.edu.sg

<http://dx.doi.org/10.1016/j.celrep.2013.09.025>

This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

SUMMARY

The pyloric epithelium continuously self-renews throughout life, driven by limited reservoirs of resident Lgr5⁺ adult stem cells. Here, we characterize the population dynamics of these stem cells during epithelial homeostasis. Using a clonal fate-mapping strategy, we demonstrate that multiple Lgr5⁺ cells routinely contribute to epithelial renewal in the pyloric gland and, similar to what was previously observed in the intestine, a balanced homeostasis of the glandular epithelium and stem cell pools is predominantly achieved via neutral competition between symmetrically dividing Lgr5⁺ stem cells. Additionally, we document a lateral expansion of stem cell clones via gland fission under nondamage conditions. These findings represent a major advance in our basic understanding of tissue homeostasis in the stomach and form the foundation for identifying altered stem cell behavior during gastric disease.

INTRODUCTION

The glandular stomach is lined by a single-cell layered epithelium that is organized into multiple gastric units resembling flask-shaped epithelial pockets. Individual gastric units comprise a glandular domain leading into a pit compartment that opens out onto the gastric surface epithelium. The gland domain is further divided into an isthmus, neck, and gland base region (Barker et al., 2010a). Two functionally distinct compartments are present in the stomach: the pyloric antrum (hereafter referred to as pylorus) opening into the small intestine, and the corpus region representing the main body of the stomach (Leushacke and Barker, 2012).

In adults, individual gastric units are considered to be homeostatically autonomous, each harboring a restricted pool of stem cells that effect tissue renewal throughout life (Nomura et al., 1998; Tatematsu et al., 1994). The turnover rate of individual gastric units differs extensively, depending on their anatomical

region and cellular composition (Lee and Leblond, 1985a, 1985b). The adult pyloric epithelium, comprising mostly mucous-secreting cells, is estimated to self-renew approximately every 10–14 days (Barker et al., 2010b). Multiple actively proliferating Lgr5⁺ cells have been recently identified to reside at the base of each pyloric gland (Barker et al., 2010b). In vivo lineage-tracing analysis determined these cells to be self-renewing, multipotent adult stem cells that contribute to epithelial renewal in the pylorus under normal homeostatic conditions (Barker et al., 2010b). Using a similar lineage-tracing approach, Arnold and colleagues identified an independent population of Sox2⁺ stem cells located within the lower region of pyloric glands (Arnold et al., 2011).

Preservation of the adult stem cell pool is essential to ensure optimal tissue homeostasis throughout adulthood. Typically, stem cells are considered to achieve a steady-state tissue homeostasis via obligate asymmetric cell divisions (Snippert and Clevers, 2011). Accordingly, each stem cell division generates two daughter cells with unequal fates: one newly derived stem cell to ensure population maintenance, and a short-lived transit-amplifying (TA) progenitor effecting epithelial renewal.

Alternatively, stochastic symmetrical divisions within the stem cell pool generating either two stem cells or two short-lived TA cells may also achieve a steady-state balance between self-renewal and tissue regeneration (Snippert and Clevers, 2011). In this model, homeostatic mechanisms have to operate at the population level to maintain constant stem cell numbers. Recently, it has been reported that homeostasis of the intestinal epithelium follows such a stochastic stem cell division behavior model. Long-term tracing analyses revealed a drift of the intestinal crypt population toward clonality over time (Snippert et al., 2010). An optimal balance between stem cell self-renewal and epithelial regeneration is therefore likely to be achieved by neutral competition between symmetrically dividing stem cells within the local niche at the base of the crypt.

The stomach recapitulates many features of the intestine, including a highly stereotypical organization and regular epithelial renewal orchestrated by Lgr5⁺ adult stem cells (Barker et al., 2010b). However, in contrast to the intestine, the mechanics of stem cell maintenance and tissue homeostasis in the stomach

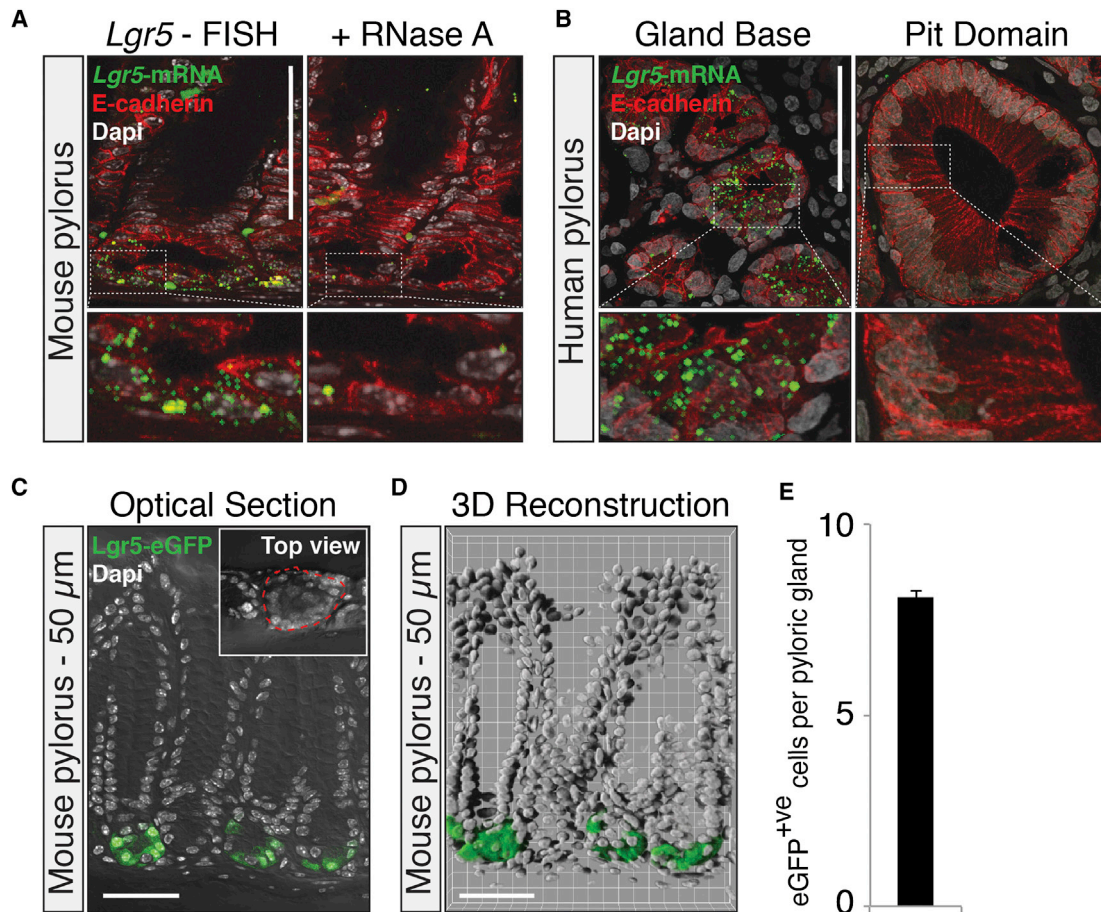


Figure 1. Expression and Proliferation Status of *Lgr5*⁺ Adult Stem Cells in the Distal Stomach

(A) Single-molecule FISH analysis for endogenous *Lgr5* expression on frozen longitudinal sections (6 μ m) of mouse pyloric epithelium. RNase A-treated tissue was used as a specificity control.

(B) As in (A), but for cross-sections of human pyloric tissue.

(C) Optical section of 50 μ m, near-native mouse pyloric section using confocal microscopy. Inset image: red dashed line circles a pyloric gland opening at the surface epithelium (top view) indicating that 50 μ m represents the average diameter of pyloric glands in mice.

(D) 3D Reconstruction from (C) using Imaris (Bitplane scientific software).

(E) Confocal imaging of pyloric glands in adult *Lgr5*-EGFP reporter mice yielded 8.1 ± 0.2 *Lgr5*-eGFP^{high} cells per gland base in the pyloric epithelium. Error bar represents SD. Scale bars represent 50 μ m. See also Figure S1 and Movie S1.

epithelium remain poorly understood. Here, we employ quantitative *in vivo* clonal fate-mapping strategies to investigate the population dynamics of *Lgr5*⁺ stem cells in the pyloric epithelium. Our findings provide unique insights into the underlying mechanisms regulating the delicate balance between stem cell maintenance and epithelial renewal during regular tissue homeostasis in the distal stomach. Understanding such mechanisms is an essential prerequisite for identifying altered stem cell behavior during gastric disease.

RESULTS

Detailed *Lgr5* Expression Analysis in Mouse and Human Pyloric Gland Epithelium

Previous studies have determined a restricted expression of *Lgr5*-driven reporters within epithelial cells located at the pyloric

gland base in the distal stomach (Barker et al., 2010b). To more accurately document the endogenous *Lgr5* expression pattern in the stomach, we performed single-mRNA molecule fluorescence in situ hybridization (FISH) for *Lgr5* within the pyloric epithelium. In agreement with independent *Lgr5* reporter mouse lines, we specifically detected endogenous *Lgr5* mRNA in cells occupying the very base of each pyloric gland (Figure 1A). Loss of signal in RNase A-treated tissue validated the specificity of the *Lgr5* FISH probe (Figure 1A). Importantly, analyses of human pyloric epithelium revealed a similar endogenous *Lgr5* expression, limited to the gland base (Figure 1B).

Using our *Lgr5*-EGFP-*Ires*-CreERT2 knockin mouse model, we next performed a detailed quantification of the *Lgr5*⁺ stem cell pool size within individual EGFP⁺ adult pyloric glands. All studies were performed on the central region of the pyloric tissue (Figures S1A, S1B, and S1C) from adult mice. Histological

analyses were performed by optical sectioning of 50- μ m-thick longitudinal sections of near-native pyloric epithelium using confocal microscopy (“xy plane side-view” imaging) (Movie S1). Fifty micrometers represent the average diameter of a pyloric gland in mouse (Figures 1C and 1D). Consequently, this imaging technique enables the visualization of the entire cell population from individual gastric units. On average, we identified 8.1 ± 0.2 Lgr5-EGFP⁺ cells in 60 EGFP⁺ glands from three different animals (Figure 1E).

Clonal Fate Mapping of Lgr5⁺ Stem Cell Output In Vivo

Lgr5⁺ cells residing at the gland base of the distal stomach were shown to actively proliferate, as determined by coexpression of *Lgr5-EGFP* and *Ki67* (Barker et al., 2010b). To investigate the proliferative history and division behavior of individual Lgr5⁺ adult stem cells in the pylorus, we adopted a short-term in vivo multicolor lineage-tracing approach. We crossed a multicolor Cre reporter mouse (Rosa four-color) to a tamoxifen-inducible *Lgr5*-driven Cre line and performed clonal fate-mapping analyses over time (Figures S2A and S2B). Following induction of Cre activity in an Lgr5⁺ cell, one of four different fluorescent reporter genes is activated at random via recombination across sets of LoxP sites (Figure S2B). Consequently, individual Lgr5⁺ cells acquire a permanent, heritable fluorescent label, allowing an accurate documentation of both short-term cell division behavior/turnover and longer-term population dynamics in their natural environment.

Initially, we validated the fluorescent expression derived from the Rosa four-color transgene in the pyloric epithelium 2 weeks postinduction (p.i.). We readily detected nuclear GFP, cytosolic YFP, cytosolic RFP, and membrane CFP-expressing cells within the pyloric epithelium, confirming a stochastic recombination behavior of the multicolor Cre reporter allele (Figure S2C). Tamoxifen-treated control animals carrying only the Rosa four-color allele showed no fluorescent expression throughout the pyloric epithelium, confirming the lack of reporter gene activity in the absence of Cre recombination (Figure S3).

Short-Term Tracing Analysis of Lgr5^{high} Cells in the Pylorus

We next examined the clonal output of single pyloric Lgr5^{high} cells over time. First, we administered a single, limiting dose of tamoxifen by intraperitoneal (i.p.) injection to adult *Lgr5-EGFP-ires-CreERT2/Rosa four-color* animals and isolated pyloric tissue 2, 4, 6, and 10 days later (Figure 2A). This dose resulted on average in one recombination event per three pyloric glands.

Individual clone sizes were quantified by counting the number of cells expressing the same fluorescent mark (derived from the four-color allele) residing in a single gastric unit. Lgr5⁺ stem cells within these clones were identified by coexpression of the *Lgr5*-driven cytosolic EGFP reporter gene. Lgr5 cell-derived nonstem cell progeny does not express *Lgr5* (Barker et al., 2010b). However, due to the half-life of EGFP, immediate non-*Lgr5*-expressing progeny carries low levels of this fluorescent label (EGFP^{low}). Such Lgr5[−] cells were incapable of generating gastric organoids in vitro (Barker et al., 2010b). For these reasons, only cytosolic EGFP^{high} cells were identified and scored as Lgr5⁺ stem cells in our analyses.

After a 2-day tracing period, more than 95% (58 out of 61) of scored Rosa four-color clones comprised a single cell coexpressing high levels of *Lgr5-EGFP*, indicating that the average Lgr5 cell division time in the pylorus exceeds 48 hr (Figure 2B). However, we also identified three two-cell clones at this time point. Whereas one clone contained no EGFP^{high} cells, the remaining two clones consisted exclusively of EGFP^{high} cells, indicating that these Lgr5⁺ cells had undergone symmetrical division to generate either two Lgr5⁺ or two Lgr5[−] cells over the 2-day tracing period.

At 4 days p.i., we identified 203 Rosa four-color clones distributed over 500 pyloric glands (Figure 2C). Approximately, 60% (122 out of 203) of the clones consisted of a single cell. The vast majority of these clones (95% [116 out of 122]) also presented high levels of cytosolic EGFP, identifying them as Lgr5⁺ stem cells (Figure 2C). A representative single Lgr5⁺ cell clone is shown in Figures 2F and 2G. The remaining single-cell clones (5% [7 out of 122]) were negative for cytosolic EGFP, indicating that these cells had lost *Lgr5*-driven *EGFP* and *Cre* expression (possibly due to migration of these cells out of the defining stem cell niche) over the 4-day period without having undergone cell division.

We also detected 81 multicellular clones, indicating that 40% (81 out of 203) of the targeted stem cells had already undergone mitosis within the 4-day period (Figure 2C). The majority of these multicellular clones comprised two cells (68% [55 out of 81]). Of note, 29 two-cell clones comprised exclusively EGFP^{high} cells, consistent with the recombined Lgr5⁺ stem cell having undergone symmetrical division to generate two *Lgr5*-expressing stem cells. We also identified 21 two-cell clones that contained no EGFP^{high} cells (Figure 2C). Accordingly, targeted stem cells forming such clones appear to have undergone symmetrical cell divisions to generate two nonstem cells. In addition to these homogeneous clones, we scored a minority of five two-cell clones that presented a mixed *Lgr5* expression, implying Lgr5⁺ cell division to generate one stem cell and one nonstem cell daughter (Figure 2C).

Importantly, we also observed 26 larger clones (13% [26 out of 203]; consisting of three or more cells) exhibiting a variety of *Lgr5* expression patterns (Figure 2C). The presence of one four-cell clone comprising exclusively EGFP^{high} cells (Figures 2H and 2I) and one eight-cell clone lacking EGFP^{high} cells (Figures 2J and 2K) further supported a predominantly symmetrical cell division behavior of Lgr5⁺ stem cells during adult pyloric gland homeostasis. (Of note, the images presented in Figures 2F, 2H, and 2J represent a single plane of a confocal z stack. The respective z stacks revealing the entire cell population are represented as Movies S2, S3, and S4.) These results also confirm earlier LacZ-based lineage-tracing results that documented a rapid contribution of at least some Lgr5⁺ stem cells to epithelial turnover in the pylorus (Barker et al., 2010b).

As expected, the size of clones comprising both Lgr5⁺ cells and their Lgr5[−] progeny (mixed clones) generally increased at later time points. At 6- and 10-day time points, we analyzed a minimum of 500 pyloric glands and documented more than 200 Rosa four-color clones (Figures 2D and 2E). However, clones containing exclusively EGFP^{high} cells (corresponding to presumptive stem cell clones) never exceeded four cells during

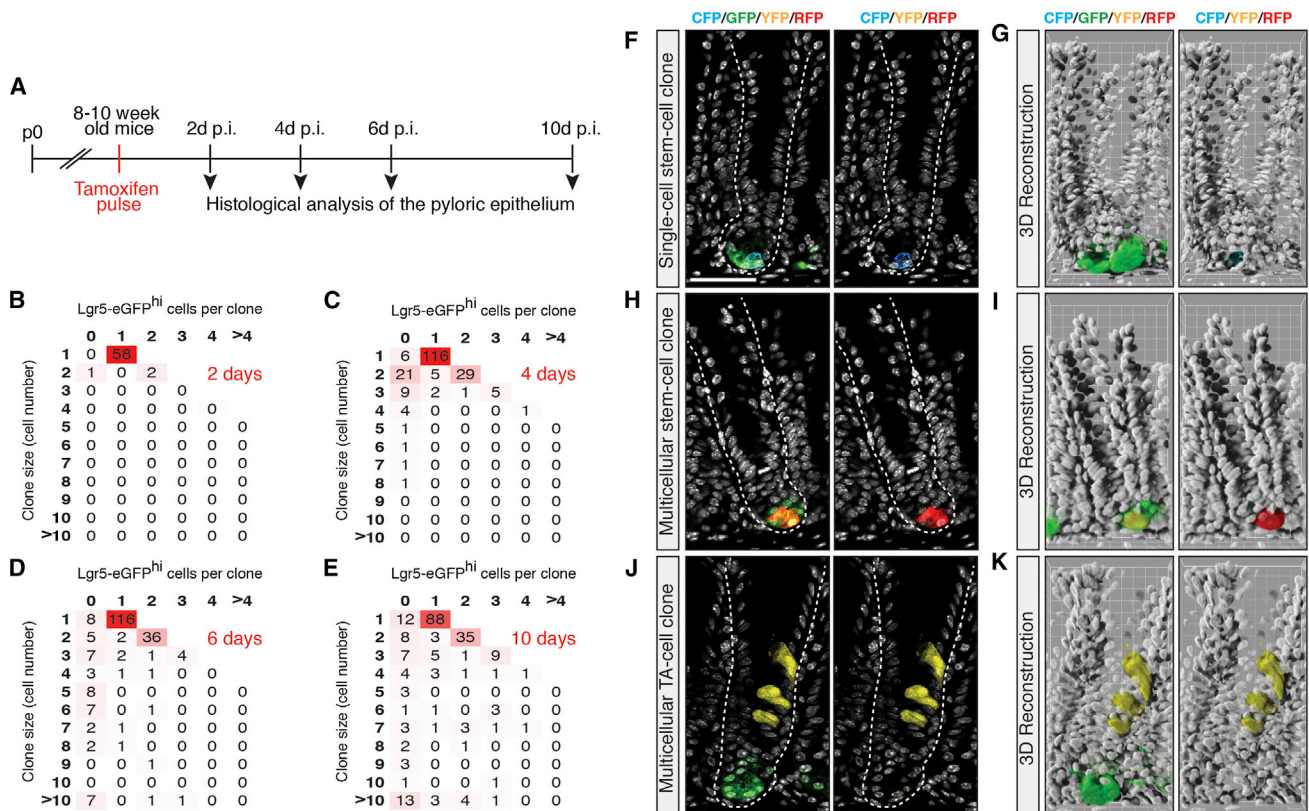


Figure 2. Short-Term Tracing Studies of Lgr5^{high} Cells in the Pylorus

(A) Experimental outline for the multicolor short-term lineage-tracing approach. Tamoxifen was given i.p. (single dose inducing on average one recombination event in three pyloric glands) to adult *Lgr5-EGFP-Ires-CreERT2* mice. Three animals were sacrificed at each indicated time point, and the endogenous fluorescence was analyzed on near-native tissues by confocal microscopy. P0, postnatal day 0.

(B) The total number of cells and Lgr5-EGFP^{high} cells were scored for each of 61 clones at 2 days post-Cre induction. The matrix indicates the absolute number of clones scored for each given clone size and given number of Lgr5^{high} cells. Red hues represent relative frequencies of all scored events: 100% is red; 0% is white.

(C) Same as (B), but after 4 days and analysis of 203 clones.

(D) Same as (B), but after 6 days and analysis of 218 clones.

(E) Same as (B), but after 10 days and analysis of 224 clones.

(F) Confocal analysis demonstrates a mCFP⁺ clone that is composed of a single Lgr5-EGFP^{high} cell.

(G) 3D Reconstruction from (F) using Imaris.

(H) RFP⁺ clone consists of exclusively Lgr5-EGFP^{high} cells.

(I) 3D Reconstruction from (H).

(J) YFP⁺ clone is composed exclusively of Lgr5-EGFP⁺ cells.

(K) 3D Reconstruction from (J).

Scale bar represents 50 μ m. See also [Figures S2–S5](#), and [Movies S2, S3, and S4](#).

this period. In contrast, multiple clones comprising exclusively EGFP^{low/-} cells (i.e., nonstem cells) rapidly expanded beyond ten cells within a 6-day period ([Figure 2D](#)). The markedly greater expansion rates of mixed/nonstem cell clones compared to the exclusive stem cell clones are consistent with a model in which relatively slowly dividing Lgr5⁺ stem cells give rise to more actively proliferating Lgr5^{low/-} TA progeny during epithelial homeostasis. Collectively, the short-term clonal analysis suggests that Lgr5⁺ stem cells predominantly divide symmetrically, producing either two Lgr5⁺ or two Lgr5⁻ cells, to maintain epithelial homeostasis in the pylorus. Additional short-term tracing Rosa four-color clone examples illustrating the clone-counting strategy are represented in [Figures S4A–S4G](#).

Mathematical Modeling of Short-Term Clonal Development

Our experimental data suggest a stochastic population model comprising Lgr5⁺ and Lgr5⁻ cells, in which approximately 90% of the Lgr5⁺ cells undergo symmetrical division into either two Lgr5⁺ or two Lgr5⁻ cells with probabilities q and $1-q$, respectively. The remaining 10% of the Lgr5⁺ cells divide asymmetrically generating one Lgr5⁺ and one Lgr5⁻ cell. We assumed Lgr5⁺ and Lgr5⁻ cells to divide on average with rates $1/t_0$ and $1/t_1$, respectively.

We applied this model to our short-term clonal data: setting $t_0 = 12$ days, in agreement with the measured single-cell clone frequency at day 10 ([Figure 2E](#)). Least-square fitting of the fraction of Lgr5⁺ cells in labeled clones at the same time point suggests an approximately equal probability for Lgr5⁺ cells to divide

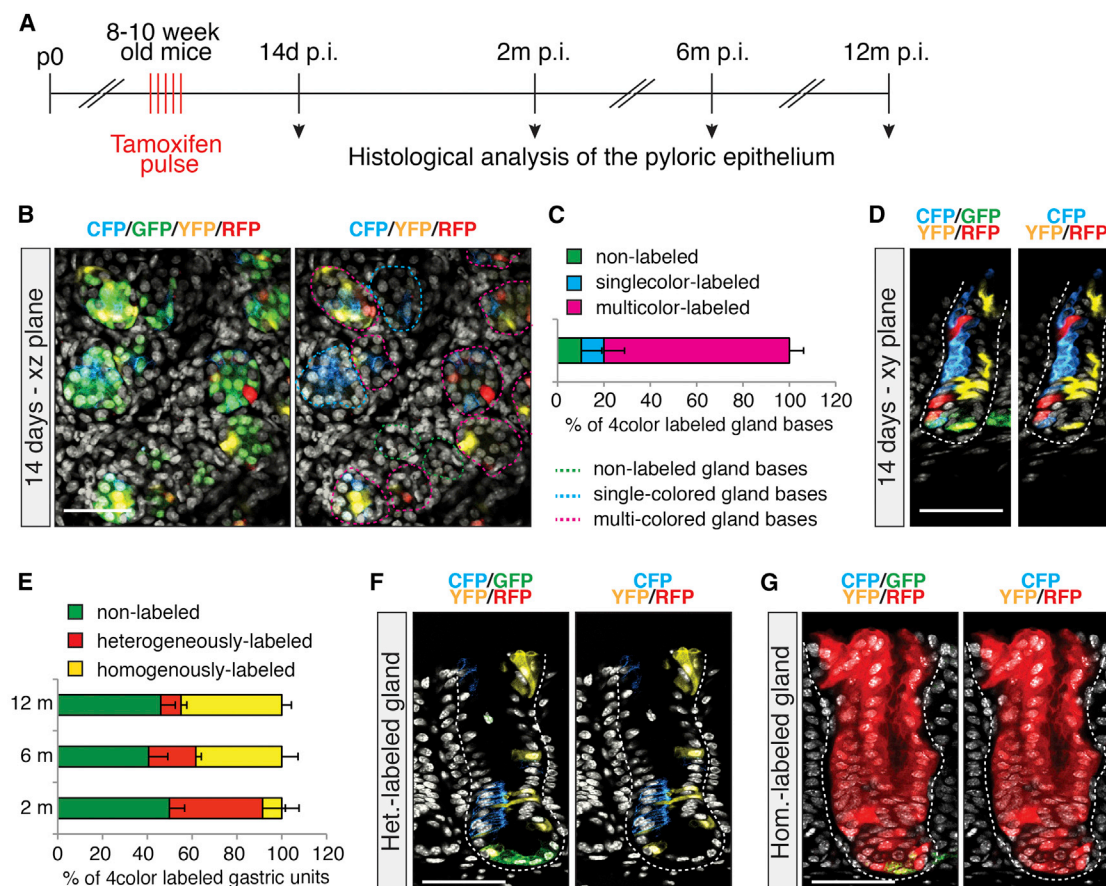


Figure 3. Long-Term Clonal Analyses of *Lgr5*⁺ Stem Cell Contributions to Homeostasis of the Pyloric Epithelium

(A) Experimental outline for the multicolor long-term lineage-tracing approach. Tamoxifen was given i.p. (five consecutive doses inducing multiple recombination events per pyloric gland) to adult *Lgr5-EGFP-Ires-CreERT2* mice. Three animals were sacrificed at each indicated time point, and the endogenous fluorescence was analyzed on near-native tissues by confocal microscopy.

(B) xz Plane image of the pyloric epithelium from the base of the gland after removal of the gastric muscle layer at 14 days p.i. Nonlabeled gland bases are marked with a green dashed line. Blue dashed line indicates gland bases carrying a single fluorescent label (derived from recombination of the four-color allele). Magenta dashed line marks multicolored gland bases.

(C) Quantification result of gland bases described in (B).

(D) xy Plane images of the pyloric epithelium at 14 days p.i.

(E) Quantification of four-color-labeled gastric units at 2, 6, and 12 months p.i.

(F) Example of a heterogeneously labeled gastric unit harboring mCFP⁺ and YFP⁺ progeny.

(G) Example of a homogeneously labeled gastric unit composed exclusively of RFP⁺ cells.

Error bars represent SD. Scale bars represent 50 μ m. See also Figure S6.

into two *Lgr5*⁺ or two *Lgr5*[−] cells ($q \approx 0.5$), as expected for a stably self-renewing stem cell population. Moreover, the average cell-cycle time for *Lgr5*[−] cells was found to be $t_1 \approx 5$ days; i.e., less than half the cell-cycle time of *Lgr5*⁺ cells.

The parameter obtained from the fit enabled predictions on the fraction of *Lgr5*⁺ cells in the labeled clones and on the clone size distribution at different times. The predictions for days 2, 4, and 6 do nicely agree with our experimental observations (Figures S5A and S5B). A prediction for day 20 is given in Figures S5C and S5D.

Long-Term Clonal Analyses of *Lgr5*⁺ Stem Cell Contributions to Homeostasis of the Pyloric Epithelium

BrdU labeling revealed the presence of multiple cycling *Lgr5*⁺ cells at the base of individual adult pyloric glands (Figure S6).

To evaluate their individual contributions to long-term tissue homeostasis, we initiated multicolor lineage tracing in multiple *Lgr5*⁺ cells residing within individual pyloric units. We administered five consecutive doses of tamoxifen by i.p. injection to adult *Lgr5-EGFP-Ires-CreERT2/Rosa four-color* mice. Confocal analyses of the clonal output were performed at 14 days, 2 months, 6 months, and 12 months after the final tamoxifen dose (Figure 3A). Confocal microscopy was performed on 50 μ m, near-native sections, as well as on whole-mount pyloric tissue, to image the epithelial layer from the base of the gland (“xz plane bottom-view” imaging) after removal of the gastric muscle layer (Movie S5). The “bottom-view” imaging technique allows the analysis of many neighboring gland bases in a single confocal stack to, facilitating the documentation of any clonal

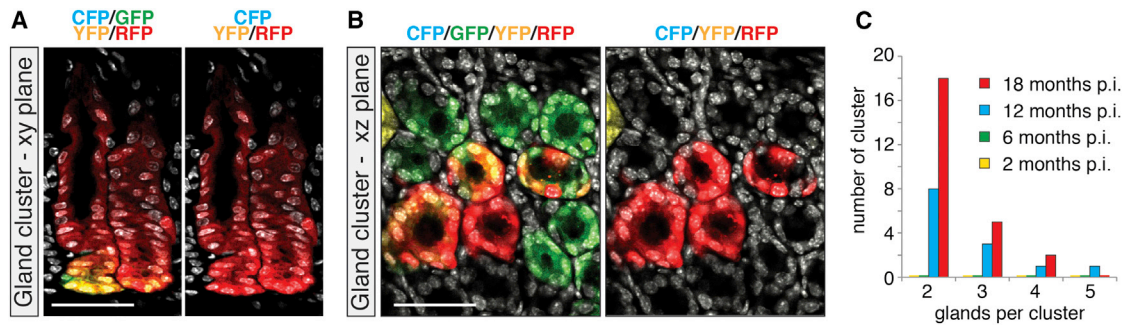


Figure 4. Lateral Expansion of Lgr5-Derived Stem Cell Clones via Gland Fission

(A) xy Plane image of the pyloric epithelium demonstrating two neighboring glands composed of exclusively RFP⁺ cells.

(B) xz Plane image of the pyloric epithelium from the base of the gland showing a cluster of RFP⁺ glands.

(C) Quantification of mono-colored gland clusters over time. Pyloric tissue presents no mono-colored gland clusters at 2 and 6 months postinduction. A total number of 13 clusters after 12 months p.i. and 25 clusters after 18 months p.i. each distributed over 400 Lgr5-EGFP⁺ glands have been identified.

Scale bars represent 50 μ m. See also [Movie S5](#).

drift within single pyloric glands toward monoclonality, or lateral expansion of clones via gland fission.

At the earliest time point (the 14-day tracing period), approximately 90% of the gland bases were labeled with a minimum of one fluorescent mark (Figures 3B and 3C). Strikingly, 90% of those recombined glands presented a heterogeneous-labeling pattern, verifying that multiple recombination events had occurred within single gastric units (Figures 3B and 3C). We readily detected four-color-labeled progeny throughout the entire epithelium of multiple gastric units (Figure 3D), validating an epithelial turnover rate of approximately 14 days in the pyloric region as reported previously (Barker et al., 2010b). After 2 months, fewer, yet larger clones were evident, with approximately 40% of Lgr5-GFP⁺ glands having lost their fluorescent four-color label, presumably due to competition between the labeled Lgr5⁺ stem cells and either unlabeled Lgr5⁺ stem cells or independent, Sox2⁺ stem cells coexisting within the same gland (Arnold et al., 2011). More than 40% of heterogeneously labeled glands, however, persisted over a period of 2 months (Figures 3E and 3F), confirming a long-term contribution of multiple Lgr5⁺ stem cells to epithelial homeostasis of single gastric units.

At later time points, we documented a general increase in tracing units contiguously spanning the entire gland/pit axis of the pyloric epithelium, consistent with the pyloric epithelium having undergone at least one complete renewal cycle. In addition, we observed a gradual reduction in the number of heterogeneously labeled glands over time (Figure 3E), consistent with neutral drift having occurred within the labeled Lgr5⁺ stem cell compartment. The first homogeneous (completely single-colored) glands were already present 2 months p.i. (Figures 3E and 3G), providing an upper estimate of the rate of neutral drift occurring within the pyloric Lgr5⁺ stem cell compartment. The frequency of this conversion to unicolor glands increased substantially to approximately 40% over the next 4 months, likely reflecting the stochastic nature of competition between labeled Lgr5⁺ stem cells (Figure 3E). This trend persisted, with 45% of labeled glands expressing a single fluorescent mark after 12 months (Figure 3E).

Lateral Expansion of Lgr5-Derived Stem Cell Clones via Gland Fission

We next examined long-term induced *Lgr5-EGFP-ires-CreERT2/Rosa four-color* mice for any evidence of clonal expansion via gland fission within the pylorus. At 2 and 6 months p.i., we did not detect any neighboring glands carrying the same Rosa four-color-derived fluorescent label. In contrast, we observed single-color gland clusters (Figures 4A and 4B) scattered throughout the pyloric epithelium after extended tracing periods. At 12 months p.i., we identified 13 clusters distributed over 400 cytosolic EGFP⁺ pyloric glands, comprising up to five neighboring glands (Figure 4C). Gland cluster counts progressively increased (up to 25 clusters over 400 cytosolic EGFP⁺ pyloric glands) after a tracing period of 18 months (Figure 4C). Collectively, this supported a lateral expansion of individual labeled units via gland fission over time. Here, gland fission is documented in the stomach under homeostatic conditions.

DISCUSSION

In vivo lineage-tracing studies demonstrated that *Lgr5* expression marks the adult stem cell populations of both the intestinal and pyloric epithelium (Barker et al., 2010b, 2007). In a follow-up study, intestinal Lgr5⁺ stem cells have been further characterized to predominantly divide symmetrically to maintain a balanced intestinal crypt homeostasis (Snippert et al., 2010). Our experimental data indicate that the majority of proliferative Lgr5⁺ stem cells in the pylorus adopt a similar stochastic strategy to the small intestine to effect epithelial homeostasis. However, a subset (7%–10%) of pyloric Lgr5⁺ stem cells does undergo asymmetric cell division in vivo, similar to what has previously been observed in the small intestine (Snippert et al., 2010). Although such asymmetric cell division may be indicative of a stem cell hierarchy existing within the Lgr5⁺ pool at the gland base, we believe that the long-term contribution of multiple Lgr5⁺ cells to epithelial homeostasis within single glandular units robustly contradicts this possibility. Instead, the distal stomach epithelium appears to be maintained by multiple

Lgr5⁺ stem cells adopting a predominantly, though not exclusive, symmetrical division mode. The ability to switch cell division behavior in vivo may endow the Lgr5⁺ stem cell compartment with the ability to quickly adapt their homeostatic activity to changes in the local environment. Although currently not technically feasible, live imaging of Lgr5⁺ cell fate decisions is needed to determine whether the cell division mode of individual Lgr5⁺ cells is a hard-wired, cell intrinsic process or if it is a truly stochastic process.

Mathematical modeling suggests that the short-term clonal dynamics of the pyloric Lgr5⁺ stem cells is consistent with a predominantly symmetrical cell division mode. The pool of Lgr5⁺ cells remains balanced because the cells were found to divide into two Lgr5⁺ stem cells or two Lgr5⁺ progenitor cells with equal probability. Such stem cell maintenance requires homeostatic control of stem cell numbers at the population level. Neutral competition of the stem cells for niche space is a possible mechanism (Snippert et al., 2010). Such an assumption, however, raises questions about the organization of the pyloric stem cell niche and the explicit homeostatic control provided by it. Our current analysis assumes a homogeneous Lgr5⁺ cell population with identical proliferation behavior of all cells. The question remains whether a quiescent subpopulation of Lgr5⁺ cells exists. Such a population could function in stress response (Yan et al., 2012), whereas having only marginal effects on the homeostatic system studied here. Answering these questions is essential to understand tissue-specific deregulation of stem cell and tissue homeostasis involved in cancer and disease.

A recent study identified Sox2⁺ cells as gastric stem cells in the pylorus. Coexpression analysis revealed *Lgr5* and *Sox2* expression to label independent stem cell populations (Arnold et al., 2011). It remains to be determined whether pyloric Lgr5⁺ and Sox2⁺ stem cells act independently (in competition) during epithelial homeostasis or if these cells exist in a hierarchy. The presence of a distinct adult stem cell population coexisting within the same epithelium is reminiscent of findings in the small intestine (Barker et al., 2010a).

Aberrant Lgr5⁺ stem cell activity in the intestine is likely to be a critical factor in driving colorectal cancer following mutation. The homeostatic behavior of Lgr5⁺ stem cells in the pylorus detailed here represents a valuable benchmark for identifying altered stem cell behavior during disease and epithelial injury in the stomach.

EXPERIMENTAL PROCEDURES

Mice

Rosa four-color mice carry the construct illustrated in Figure S3A. For the Rosa four-color construct, refer to Snippert et al. (2010). Rosa four-color mice were bred to *Lgr5-EGFP-Ires-CreERT2* mice as described previously (Barker et al., 2007). All animal experiments were approved by the Institutional Animal Care and Use Committee of Singapore.

Tamoxifen Administration

Short-term tracing Cre induction was performed in 8- to 10-week-old mice that were injected i.p. with a single dose of 0.15 mg tamoxifen/g body weight. Long-term tracing Cre induction was performed in 8- to 10-week-old mice that were injected daily with doses of 0.1 mg tamoxifen/g body weight on 5 consecutive days.

Single-Molecule FISH

The single-molecule FISH procedure employed here was described earlier (Barker et al., 2012) and according to manufacturer instructions (Biosearch Technologies). RNase A treatment was performed subsequent to the postfixation step of the tissue sections. Sections were incubated for 45 min at 37°C at a concentration of 100 µg/ml RNase A (Qiagen) in PBS. Human tissue samples were provided by the NUHS Tissue Repository, Singapore.

Tissue Preparation for Optical Sectioning by Confocal Microscopy

“xy plane” side-view imaging was performed using semithick sections of near-native tissue, generated as described in Snippert et al. (2011). Once solid, a vibrating microtome was used to perform semithick longitudinal sections (50 µm) of the pyloric epithelium. Sections were directly mounted into Hydromount (National Diagnostics) containing Hoechst dye nuclear counterstain.

“xz plane” bottom-view imaging was performed after removal of the muscle layer, and the epithelial tissue was directly mounted upside down in Hydromount containing Hoechst dye nuclear counterstain.

Confocal Microscopy

Images were acquired using an Olympus FV1000 upright confocal microscope. For the Rosa four-color imaging, scans were performed in a series for fluorescent protein excitation as described in Snippert et al. (2011).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and five movies and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2013.09.025>.

ACKNOWLEDGMENTS

We thank Graham Wright and John Lim for their imaging assistance, Valerie Hu and Gurmit Singh for their mouse husbandry work, and Casslynn Koh for her excellent histology work. N.B. is supported by the Singapore Gastric Cancer Consortium.

Received: July 19, 2013

Revised: August 23, 2013

Accepted: September 17, 2013

Published: October 24, 2013

REFERENCES

- Arnold, K., Sarkar, A., yram, M.A., Polo, J.M., Bronson, R., Sengupta, S., Seandel, M., Geijsen, N., and Hochedlinger, K. (2011). Sox2(+) adult stem and progenitor cells are important for tissue regeneration and survival of mice. *Cell Stem Cell* 9, 317–329.
- Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P.J., and Clevers, H. (2007). Identification of stem cells in small intestine and colon by marker gene *Lgr5*. *Nature* 449, 1003–1007.
- Barker, N., Bartfeld, S., and Clevers, H. (2010a). Tissue-resident adult stem cell populations of rapidly self-renewing organs. *Cell Stem Cell* 7, 656–670.
- Barker, N., Huch, M., Kujala, P., van de Wetering, M., Snippert, H.J., van Es, J.H., Sato, T., Stange, D.E., Begthel, H., van den Born, M., et al. (2010b). Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell Stem Cell* 6, 25–36.
- Barker, N., Rookmaaker, M.B., Kujala, P., Ng, A., Leushacke, M., Snippert, H., van de Wetering, M., Tan, S., Van Es, J.H., Huch, M., et al. (2012). Lgr5(+ve) stem/progenitor cells contribute to nephron formation during kidney development. *Cell Rep.* 2, 540–552.
- Lee, E.R., and Leblond, C.P. (1985a). Dynamic histology of the antral epithelium in the mouse stomach: II. Ultrastructure and renewal of isthmal cells. *Am. J. Anat.* 172, 205–224.

- Lee, E.R., and Leblond, C.P. (1985b). Dynamic histology of the antral epithelium in the mouse stomach: IV. Ultrastructure and renewal of gland cells. *Am. J. Anat.* 172, 241–259.
- Leushacke, M., and Barker, N. (2012). Lgr5 and Lgr6 as markers to study adult stem cell roles in self-renewal and cancer. *Oncogene* 31, 3009–3022.
- Nomura, S., Esumi, H., Job, C., and Tan, S.S. (1998). Lineage and clonal development of gastric glands. *Dev. Biol.* 204, 124–135.
- Snippert, H.J., and Clevers, H. (2011). Tracking adult stem cells. *EMBO Rep.* 12, 113–122.
- Snippert, H.J., van der Flier, L.G., Sato, T., van Es, J.H., van den Born, M., Kroon-Veenboer, C., Barker, N., Klein, A.M., van Rheenen, J., Simons, B.D., and Clevers, H. (2010). Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. *Cell* 143, 134–144.
- Snippert, H.J., Schepers, A.G., Delconte, G., Siersema, P.D., and Clevers, H. (2011). Slide preparation for single-cell-resolution imaging of fluorescent proteins in their three-dimensional near-native environment. *Nat. Protoc.* 6, 1221–1228.
- Tatematsu, M., Fukami, H., Yamamoto, M., Nakanishi, H., Masui, T., Kusakabe, N., and Sakakura, T. (1994). Clonal analysis of glandular stomach carcinogenesis in C3H/HeN<=>BALB/c chimeric mice treated with N-methyl-N-nitrosourea. *Cancer Lett.* 83, 37–42.
- Yan, K.S., Chia, L.A., Li, X., Ootani, A., Su, J., Lee, J.Y., Su, N., Luo, Y., Heilshorn, S.C., Amieva, M.R., et al. (2012). The intestinal stem cell markers Bmi1 and Lgr5 identify two functionally distinct populations. *Proc. Natl. Acad. Sci. USA* 109, 466–471.